PATENTS
Atty. Docket No. VOS-012

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant:	Oliver Bruestle	Art Unit:	1632
Serial No.:	09/581,890	Examiner:	Falk, Anne Marie
Filing Date:	August 28, 2000		
Title:	**Neural Precursor Cells, Method for the Production and Use Thereof in Neural Defect Therapy**		

Mail Stop AF
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

DECLARATION OF DR. OLIVER BRUESTLE UNDER 37 C.F.R. § 1.132

Dear Sir:

I, Oliver Bruestle, declare as follows:

1. I am the inventor of the invention claimed in the above-referenced patent application.

2. I obtained my M.D. in 1989 from the University of Ulm Medical School, Germany. I worked as a resident in neuropathology at the University of Zurich, Switzerland, and as a resident in neurosurgery at the University of Erlangen-Nürnberg, Germany. I performed a postdoctoral fellowship at the National Institutes of Health, Bethesda, MD in the area of stem cell research and was group leader in neuropathology at the Univeristy of Bonn Medical Center. Since 2002 I have served as the Director of the Institute of Reconstructive Neurobiology at the University of Bonn Medical Center, Bonn, Germany. I have authored or co-authored several papers regarding

neurobiology and neural precursor cells. My curriculum vitae including a selected list of these publications is enclosed herewith as Appendix A.

3. Currently, I am the Director of the Institute of Reconstructive Neurobiology at the University of Bonn Medical Center in Bonn, Gemany.

4. I am familiar with the above-referenced patent application, the Office Action dated February 26, 2003, and the Okabe *et al.* reference cited in the Office Action.

5. My understanding is that the 08/581,890 application has claims, as amended, directed to cell compositions derived from embryonic stem cells. The composition comprises from 85% to 100% isolated neural precursor cells, which have the ability to differentiate into neuronal cells, glial cells, or combinations thereof, and further comprising from 0% to 15% primitive embryonic and non-neural cells, and the cell composition is non-tumorigenic. The Office Action alleges that the application does not reasonably provide enablement for a composition comprising from 85% to 100% isolated neural precursor cells. (Office Action, page 2) I disagree with this statement. The Office Action also alleges that the cell compositions disclosed by Okabe *et al.* are indistinct from those instantly claimed. (Office Action, page 4) I also disagree with this statement.

6. The data presented below demonstrates that the compositions of the present invention contain neural cells in purities approaching or reaching 100%. The presence of undifferentiated, tumorigenic cells has been essentially eliminated in the cell compositions of the present invention.

7. It is common knowledge that transplantation of undifferentiated ES cells carries a high risk of tumor formation (Damjanov, *Int. J. Dev. Biol.* 37:39–46, 1993, attached hereto as Appendix B). Grafted ES cells typically give rise to teratomas and teratocarcinomas, *i.e.* tumors which are composed of a variety of cell types and tissues of several germ layers. Teratoma induction is an established assay to document the

pluripotency of newly generated ES cell lines (Thomson *et al., Science* 282:1145-47, 1998, attached hereto as Appendix C).

8. A key prerequisite for the therapeutic application of donor cells derived by *in vitro* differentiation of ES cells is the elimination of any residual undifferentiated ES cells, which could give rise to tumors. Previous methods were not suitable to generate ES cell-derived neural cells in such a purity. The method described by Okabe *et al.* (*Mech. Dev.* 59:89-102, 1996), cited in the Office Action, includes the aggregation of ES cells to embryoid bodies, plating of the embryoid bodies in ITSFn medium and subsequent proliferation of neural precursors in the presence of FGF2 ("N3FL medium"). This method is not suitable to generate purified, non-tumorigenic cell populations because it does not eliminate sufficient levels of residual undifferentiated ES cells to create a non-tumorigenic cell composition. In contrast, the cell compositions of the present invention are essentially devoid of undifferentiated ES cells and, following transplantation, do not generate tumors.

9. Alkaline phosphatase is an enzyme typically expressed in undifferentiated ES cells. The enzyme can be easily identified by histochemistry. I note that cell populations generated by the method of Okabe *et al.* still contain a significant fraction of undifferentiated alkaline phosphatase-positive cells. Even under the most optimal conditions, cells propagated in N3FL still contain alkaline phosphatase-positive cells (*See* Fig. 1 A-B and Fig. 2 below). These cells typically grow in small colonies which enlarge over time. In contrast, cells subsequently propagated in the presence of EGF+FGF2 or EGF+FGF2 followed by FGF2+PDGF (according to the specification of the application) contain no alkaline phosphatase-positive colonies. This is shown in Fig. 1 C-F and Fig. 2 below.

Fig. 1: Alkaline phosphatase histochemistry in ES cell-derived neural precursors.

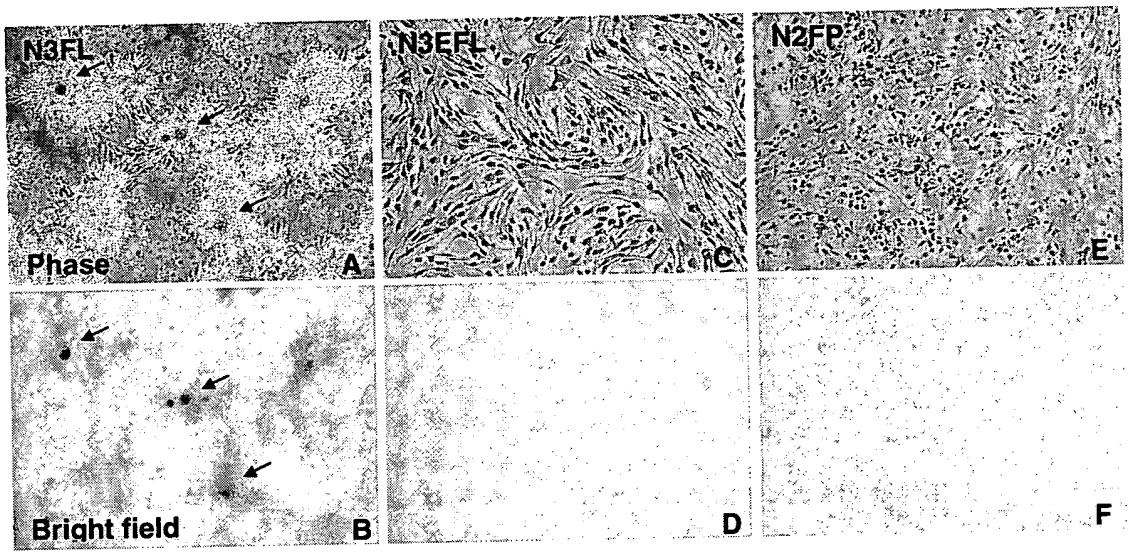


Fig. 1: A-B: ES cells aggregated to embryoid bodies, plated in ITSFn medium and subsequently propagated in N3FL medium for 5 days yield neural precursor cell populations which still include colonies of alkaline phosphatase-positive undifferentiated ES cells (Okabe method; A and B). **C-F**: ES cell-derived neural precursors further propagated in the presence of FGF2+EGF (N3EFL; **C-D**) or FGF2+EGF followed by FGF2+PDGF (N2FP; **E-F**) are devoid of undifferentiated alkaline phosphatase-positive cells. Phase contrast and bright field pictures are depicted in the upper and lower panel, respectively.

10. The presence of alkaline phosphatase-positive cells correlates with the tumorigenicity of cells generated by the Okabe method. As mentioned in the statement of Dr. Johan Bengzon, attached hereto as Appendix D, "[u]pon transplantation into the rat brain, cells grown under these conditions consistently gave rise to non-neural adenoid tissue and tumors." The statement of Dr. Bengzon further indicates that since the purity of the precursor cell populations generated was not suitable for transplantation, the study was discontinued.

11. The observations of Dr. Bengzon, as described in his statement, are consistent with our own data. As discussed above, it is common knowledge to a skilled neurologist that ES cells transplanted at an undifferentiated stage can form tumors. In addition, aberrantly differentiated ES cells may generate foreign tissues in the graft.

Even a small number of undifferentiated or aberrantly differentiated ES cells suffices to cause these problems. (Björklund *et al.*, *PNAS* 99:2344-2349, 2002, attached hereto as Appendix E). Following transplantation of cells generated according to the method of Okabe *et al.* into the ventricle of embryonic rats, we find a large proportion of tumorous clusters. This is true for both cells propagated in ITSFn medium (Brüstle *et al.*, *PNAS* 94:14809-14, 1997, attached hereto as Appendix F) and cells propagated in FGF2 (N3FL medium) (Wernig *et al.*, manuscript submitted for publication, attached hereto as Appendix G). In contrast, the transplant studies described in the patent application as well as subsequent publications in high ranking journals (*e.g.*, Brüstle *et al.*, *Science* 285:754-756, 1999, attached hereto as Appendix H) clearly demonstrate that the cell compositions of the present invention do not show the formation of tumors or non-neural tissues when transplanted into the central nervous system. The formation of tumors and non-neural tissues is the most sensitive criterion used to judge the purity of grafted ES cell-derived neural precursors.

12. The fact that grafted neural precursors generated according to the present invention yield cell compositions that can be transplanted without evidence of the formation of tumors or non-neural tissues demonstrates that neural cells were generated in purities approaching or reaching 100%.

13. This information provides an important difference between the cell compositions of the present invention, which are non-tumorigenic, and those of Okabe *et al.*, which are tumorigenic.

14. Furthermore, as described below, the compositions of the present invention contain an increase in cells expressing markers of early neural cells and leads to an overall enrichment of neural cells to levels approaching or reaching 100%.

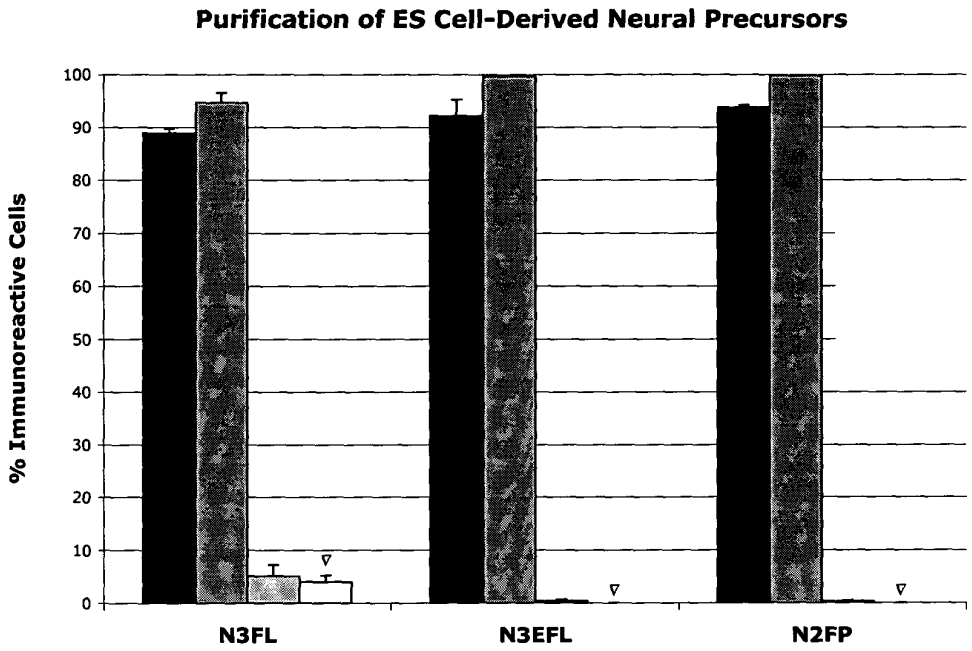
15. A neural precursor cell is defined by its ability to generate mature neural cells, *i.e.*, glial or neuronal cells. There are no unequivocal markers for defining neural

precursor cells. There are, however, markers which are broadly, yet not exclusively, expressed in neural precursors. For example, nestin is a marker for early neural cells that is also expressed in astrocytes, endothelial cells and microglial cells (*i.e.*, a cell population originating from hematopoietic cells). (Sotelo *et al.*, *J. Neurosci.*, 14(1):124-133, 1994, attached hereto as Appendix I). Nestin and the surface antigen A2B5 are most commonly used as markers of early neural cells. As shown in Fig. 2, the compositions of the present invention have an increase in the number of cells expressing either or both of these marker antigens.

16. Even under the most optimal conditions, precursor cell cultures tend to undergo some degree of spontaneous differentiation into more mature daughter cells. The ability to differentiate into mature neuronal and glial cells is, in fact, a key property of neural precursor cells. Therefore, a comprehensive analysis of the purity of a neural precursor cell population has to include markers for the fraction of spontaneously differentiated progeny.

17. We have applied a cocktail of antibodies to markers of both immature (nestin and A2B5) and differentiated (beta-III-tubulin, GFAP, and 04) neural precursor cells in order to determine the overall purity of the generated neural cells. Our data (shown below) demonstrates that the cell compositions of the present invention contain more than 99% neural cells.

Fig. 2: Expression of neural markers and alkaline phosphatase (AP) in ES cell-derived neural precursor cell populations.



	N3FL (FGF2)	N3EFL (FGF2+EGF)	N2FP (FGF2+EGF followed by FGF2+PDGF)
Nestin ± A2B5	88.89 ± 0.79	92.18 ± 3.04	93.77 ± 0.45
Neuro-Cocktail +	94.67 ± 1.85	99.56 ±0.39	99.70 ± 0.22
Neuro-Cocktail -	5.08 ± 2.08	0.38 ± 0.33	0.27 ± 0.23
AP +	3.94 ± 1.23	0	0

Fig. 2: ES cells aggregated to embryoid bodies, plated in ITSFn medium and subsequently propagated in N3FL medium (Okabe method) and ES cell-derived neural precursors subsequently propagated in the presence of FGF2+EGF (N3EFL) or FGF2+EGF followed by FGF2+PDGF (N2FP) were subjected to alkaline phosphatase histochemistry or immunofluorescence analysis. Cells were co-stained with antibodies to nestin and A2B5 or a 'Neuro-Cocktail' including antibodies to nestin, A2B5, beta-III-tubulin (a neuronal marker), GFAP (an astrocytic marker) and 04 (an oligodendroglial marker). Results for nestin ± A2B5 staining include cells stained by either or both nestin and A2B5. Note the disappearance of AP-positive cells and the increase of the nestin and/or A2B5-positive cell fraction. In stages N3EFL and N2FP, virtually all cells express the neural marker antigens covered by the "Neuro-Cocktail" whereas the unlabeled cells ("Neuro-Cocktail –") approach 0%. Data are based on 3 independent experiments. For immunofluorescence analysis, at least 1,000 cells were quantified for each condition. Quantification of AP-positive colonies is based on 20 high power fields per condition and experiment.

18. Additionally, the fact that Dr. Bengzon and other scientists collaborating with Dr. Okabe have abandoned transplantation of cells generated according to the Okabe method (as indicated in the statement of Dr. Bengzon) illustrates that the cell compositions of the present invention represent an unexpected breakthrough.

19. Therefore, these data clearly demonstrate that undifferentiated, tumorigenic ES cells have been essentially eliminated from the cell compositions of the present invention and neural precursor cells have been generated in unprecedented purity approaching 100%. Accordingly, it is my opinion that the specification as filed enables the skilled neurologist to make non-tumorigenic cell compositions derived from embryonic stem cells in which the composition comprises from 85% to 100% isolated neural precursor cells that have the ability to differentiate into neuronal cells, glial cells, or combinations thereof.

20. Additionally, these data and the statement of Dr. Bengzon clearly demonstrate that the cell compositions of the present invention are non-tumorigenic whereas the cell compositions of Okabe *et al.* are tumorigenic. Accordingly, it is my opinion that the claimed cell compositions are different from those described in Okabe *et al.*

I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true and further that these statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Date _____

_____
Dr. Oliver Bruestle